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^{14}C-SULFUR MUSTARD ADDUCTS OF CALF THYMUS DNA

Sidney Yaverbaum

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U. S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE
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A grant was awarded to TNO-FML to develop immunochemical monitoring systems for the detection of DNA-HD and Protein-HD adducts in humans following exposure to HD. TNO-PML has been using 35S-HD to prepare adducts for their assays, which have inherent shortcomings that limit detection sensitivity. An experimental batch of 14C-HD-DNA adducts was prepared in an attempt to increase the assay sensitivity. Double- and single-stranded purified calf thymus DNA preparations were reacted with 142, 14.2 and 1.42 uM of 14C-HD under aqueousfree conditions. The 14C-HD-DNA adducts were isolated at -20C in 75% ethanol solution and freed of HD agent and organic solvents (i.e., acetone and alcohol). The 14C-HD-DNA adducts in aqueous buffer were analyzed for specific activity and purity. The ds-DNA-HD adducts were uncontaminated, but the ss-DNA-HD adducts were initially slightly contaminated with alcohol. The 14C-HD-DNA adducts will be compared to 35S-HD-DNA adducts similarly prepared by TNO-PML at their facilities to determine which of the two radioisotope DNA adducts will yield more sensitive and reliable immunochemical assays for the detection of DNA-HD adducts.

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PREFACE

This protocol was performed to determine the potential advantage of replacing [^{35}S]HD-DNA and [^{35}S]HD-Protein reagents with [^{14}C]HD-DNA and [^{14}C]HD-Protein reagents for increasing the sensitivity of the immunochemical detection assay for HD-DNA and HD-Protein adducts under development at TNO-PML.

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INTRODUCTION

A contract was awarded to TNO-PML to develop immunochemical monitoring systems for the post-exposure detection of sulfur mustard (HD) adducts in human white blood cell (WBC) DNA and blood proteins (hemoglobin and albumin) (1). Nucleoside adducts were isolated and identified from [^{35}S]DNA of WBCs exposed to [^{35}S]HD, the adducts were characterized and synthesized, antisera were produced against them, and a direct ELISA test was developed for the detection of adducts by polyvalent and monovalent antibodies (1). The same developmental scheme is in progress with respect to [^{35}S]HD-Hb (hemoglobin) and [^{35}S]HD-Alb (albumin) adducts.

Analyses of results suggested that synthesized protein-HD adducts may not be good antigenic moieties when conjugated to immunogenic carrier proteins, or that the [^{35}S]-HD is not sensitive enough because the specific activity is lower than required. Another reduction in sensitivity occurs as ^{35}S approaches and passes its half-life of 88 days. The scientists at TNO-PML compared their [^{35}S]HD with a [^{14}C]HD preparation listed in MRICD's inventory as containing 41 mCi/mM HD. They calculated that the latter had more than twice the specific activity of the former and a half-life (5700 years) that assured no loss of sensitivity over time.

It is anticipated that the DNA-HD adduct immunochemical detection system will be brought in-house. As one aspect of the transfer of technology, it is advantageous to prepare [^{14}C]HD-DNA by the same procedures used by TNO-PML for comparison with [^{35}S]HD-DNA to determine if there are any increases in sensitivity and reliability. Since the immunochemical tests are currently in progress at TNO-PML, the radiolabeled carbon derivatives of DNA (i.e., DNA-HD[^{14}C] adducts) will be shipped there for evaluation.

MATERIALS AND METHODS

A. Experimental Design

Solutions (0.848 mg/ml) of calf thymus double- (ds-) and single-stranded (ss-) DNA were reacted with dilute concentrations of [^{14}C]HD (i.e., 142, 14.2, and 1.42 uM) in dry acetone for 60 min at 37°C, and then cooled down in ice. A sodium acetate-EDTA buffer, pH 5.5 was added and the mixture shaken. The DNA was precipitated with three volumes of absolute alcohol at -20°C. The DNA was collected on glass rods, washed with 80% ethanol, dried in air, and dissolved in Tris Buffer (10 mM Tris-HCl, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$, 0.2 mM $ZnSO_4$, pH 7.2) overnight at room temperature. All traces of alcohol were removed from ss-DNA-HD precipitates by gently blowing dry nitrogen gas over the surface of the compounds before dissolution in buffer. The solutions

were left overnight to dissolve in Tris Buffer at room temperature with rotary stirring. DNA in each preparation was measured in a spectrophotometer, diluted to ~ 1 mg/ml, and subsequently stored at -20°C.

B. Methods

Details of the procedures described in this pilot protocol were those currently used at TNO-PML for the preparation of DNA-HD[^{35}S] adducts (Appendix A). The directives were followed as stated although the stock HD reagents and alkylating volumes were different.

1. Preparation of DNA Solutions. Double-stranded calf thymus DNA (Sigma, D 1501, Type I, lot 67F-9730) was soaked in distilled water overnight (>20 mg/ml). The ds-DNA was dissolved in phosphate buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 15 mM KH_2PO_4, adjusted to pH 7.4 with 50% NaOH) to a concentration of about 2 mg/ml with overnight rotary stirring at room temperature. The concentration of ds-DNA was measured in a Shimadzu UV-260 spectrophotometer (A_{260}, 1 mg/ml=20) and diluted to ~ 1 mg/ml in PBS. The solutions were stored in the refrigerator at 4°C for 18 hrs until used.

Single stranded DNA was prepared by heating half the DNA solution for 10 minutes at 100°C in a closed heat-resistant screw-capped vial. The solution was rapidly quenched on ice and stored in the refrigerator.

Prior to treatment with XHD solutions, the ds- and ss-DNA solutions were divided into four equal volumes in screw-capped glass scintillation vials.

2. Preparation of XHD Solutions. The only useful [^{14}C]HD preparation available was T-19-85-/53. After DNA treatment was initiated it was shown to contain 0.072 mCi/mg HD, and 180 mg HD/ml (11.45 mCi/mM HD). It was diluted in an exclusion area surety laboratory in dry acetone in a clean, dry, small vial as follows: 5 ul [^{14}C]HD added to 791 ul dry acetone yielded a 7.1 uM HD solution (i.e, 0.11% or 1.131 mg/ml).

The 7.1 uM HD solution was taken to a hood approved for radioactive dilute agent use. The solution was diluted 1:10 (50 ul of 7.1 uM HD + 450 ul dry acetone) and 1:100 (50 ul of 0.71 uM HD + 450 ul dry acetone) to yield 0.71 and 0.07 uM solutions, respectively.

3. Treatment of DNA with XHD. The three XHD solutions, 7.10, 0.71, and 0.07 uM, were used to treat one each of the ds- and ss-DNA solutions by the addition of 20 ul of radiolabeled XHD for each 1 ml of DNA solution. This is equivalent to treatment concentrations of 17.137, 1.123, and 0.128 x 10^{-3} mg HD/3.986 mg

ds-DNA/4.7 ml solution, and 16.402, 1.075, and 0.123 x 10^{-3} mg HD/3.816 mg ss-DNA/4.5 ml solution. Dry acetone was added to the fourth ds-and ss-DNA solutions for controls. The solutions were stirred briefly on a vortex mixer and incubated in a water bath for 60 min at 37°C.

4. Purification of [^{14}C]HD-DNA. The DNA-XHD solutions were placed in a bucket of ice chips after the 1 hr incubation at 37°C and remained there for the next three steps. An aliquot of cold acetate-EDTA buffer (3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5) equal to 0.1 volume of each DNA-HD solution was added with stirring. The DNA in each solution was precipitated with the addition of three volumes of -20°C absolute ethanol.

The ds-DNA was collected with a glass rod, washed with two 2.0 ml aliquots of ice cold 80% ethanol, dried for 2 min in air, and then dissolved at about 1 mg/ml in Tris Buffer overnight with rotary stirring at room temperature.

The ss-DNA, still in 75% cold ethanol, was kept overnight at 4°C.

The concentrations of ds-DNA solutions were determined spectrophotometrically (A_{260}, 1 mg/ml=20) after a 16- to 18-hr overnight incubation, and the solutions stored at -20°C.

The ss-DNA solutions were centrifuged and pelleted (15 min; 4°C; 600g). The supernatants were carefully aspirated, and the last bit of alcohol removed by a gentle stream of dry N_2 gas. The ss-DNA wet precipitates were dissolved at or about 1 mg/ml in Tris Buffer overnight with rotary stirring at room temperature.

Concentrations of the ss-DNA solutions were measured spectrophotometrically (A_{260}, 1 mg/ml=26 for ss-DNA) after a 16- to 18-hr incubation, and the DNA solutions stored at -20°C.

5. Data Collection. Radioactivity of the alkylated solutions was measured in a Beckman LS 5801 scintillation counter. Duplicate aliquots of 0.10 ml were placed in glass scintillation vials to which 10.0 ml of Ready Safe scintillation fluid (Beckman) was added and then shaken. An average disintegrations per minute (DPM) was obtained for a 5-minute count, with appropriate instrument and sample background controls.

DNA concentrations of the solutions were measured in a Shimadzu UV-260 spectrophotometer at 260 nm and over a range of 320 to 220 nm.

C. Data Analysis

The DNA and radioactivity contents of the alkylated solutions were determined, and the specific activity of each preparation calculated. The solutions were scanned over a range of 320-220 nm, and the purity determined for each as follows.

DNA Purity. The ratio of absorbance at 260 nm divided by absorbance at 280 nm. This is a standard DNA analysis, with the ratio from 1.8 to 1.9 indicative of purified DNA.

Presence of residual ethanol on DNA. The ratio of absorbances at 230 nm/260 nm (Ms. Scheffer, TNO-PML, personal communication). This ratio is used at TNO-PML to indicate the absence or presence of alcohol in DNA preparations (0.4 to <0.5, and ≥0.5, respectively).

RESULTS

The specific activities of alkylated ss-DNA (0.90 - 65.90 nCi/mg DNA) were considerably higher than those of ds-DNA (0.04 - 22.44 nCi/mg DNA) at the same HD concentrations (Table I). The ss-DNA-HD and ds-DNA-HD alkylated preparations showed a linear response to the mustard treatments (r=0.999998 and 0.999660, respectively)(Table I, Fig. 1). The efficiency of alkylation for ds-DNA preparations ranged from 1.58 - 6.64% (r=0.456443) and from 8.90 - 16.77% for ss-DNA preparations (Table III). Similarly, the data show that with decreasing HD concentrations the alkylating efficiency of HD appears to be a hard reverse for ss-DNA-HD solutions (r=-0.996034) and not very good for ds-DNA-HD solutions (r=0.456443) (Table III, Fig. 2).

The ds-DNA-HD preparations were free of protein as evidenced by their 260 nm/280 nm ratios of 1.8 - 1.9. Single stranded DNA-HD preparations were slightly grainy and contained some impurity (Table II; 260 nm/280 nm). The 230 nm/260 nm ratio indicates that residual alcohol may be present (the only substance to come in contact with DNA other than aqueous buffers) that may be affecting the solubility of the ss-DNA preparations (Table II).

DISCUSSION/CONCLUSION

The [^{14}C]HD-treated DNAs were prepared by the procedure described by TNO-PML. The alkylated ds-DNA preparations appear to be pure by the data analysis criteria. The ss-DNA and ds-DNA preparations generally alkylated in a [^{14}C]HD concentration-dependent manner; i.e., the higher the [^{14}C]HD radioactivity applied, the higher the DNA-HD product radioactivity (Table I, Fig. 2). The reason for lower alkylation efficiencies at the higher [^{14}C]HD concentrations is unclear (Table III).

Equally unclear are the depressed 260 nm/280 nm ratios of the alkylated ss-DNA solutions, because extraneous protein was never used in the procedure. DNA No. D-1501 from Sigma has ≤ 3% protein contamination. Using serum albumin as the protein standard (A_{280}, 1 mg/ml = 39.6) and an average of 0.788 mg ds-DNA/ml, will yield a maximum of 0.024 mg protein/ml of solution. At a 1:20 dilution this equates to 0.047 OD units/ml (1.0 mg protein/ml at a 1:20 dilution = 1.98 OD units). Similarly, an average of 0.426 mg ss-DNA/ml will yield 0.013 mg protein/ml of solution. At a 1:26 dilution this equates to 0.020 OD units/ml (1.0 mg protein/ml at a 1:26 dilution = 1.523 OD units). If subtracted from the A_{280} measurements, they would increase ds-DNA and ss-DNA 260:280 ratios by 0.2 and 0.1, respectively. This would run the ds-DNA 260:280 nm ratio over 2.0, which means that there is considerably less protein present. Neither of these OD adjustments at A_{280} significantly affects the group results. It is possible that residual alcohol is affecting ss-DNA-[^{14}C]HD configuration (the solutions have a colloidal appearance) to a degree that the 260 nm/280 nm ratio is affected. The alkylated ss-DNA solutions can be rectified by reprecipitation of the compounds and total elimination of residual alcohol with dry N_2 gas during the repurification procedure.

The maximum alkylation reactions for ds-DNA and ss-DNA were 6.64% and 16.77%, respectively, i.e., the radioactivity of the [^{14}C]HD treatment vs. the radioactivity of the [^{14}C]HD-DNA complexes. Benschop *et al*. reported that 5-15% of the alkylating agent reacted with DNA (1989). The alkylating efficiencies obtained in the two laboratories are approximately equal. The higher alkylation percentages for ss-DNA are due to increased exposure of purines and phosphorylated pentoses to [^{14}C]HD.

There are sufficient positive results to warrant repeating the experiment under more optimal conditions. The conditions under which this experiment was performed used a surety agent that contained 11.45 mCi/mM HD, which is approximately 1/4 the optimal specific activity required. The surety agent on inventory at MRICD, which contains undiluted 41 mCi/mM HD, has been reconstituted with methylene chloride and is currently being analyzed by Dr. C.A. Broomfield (personal communication). Starting with this level of specific activity and undiluted agent, the procedure should yield higher test adduct antigen specific activities that would increase sensitivity of the HD-DNA and HD-protein adduct immunochemical detection assay.

Table I

RADIOACTIVITY OF [^{14}C]HD-ALKYLATED DNA*

Preparation Code**	M (± SD) DPM / mg DNA @	nCi/mg DNA	x 10^{-6} mg[^{14}C]HD / mg DNA #
ds-DNA-142	49,809 (± 64.9)	22.44	311.92
ds-DNA-14.2	3,226 (± 4.14)	1.45	20.16
ds-DNA-1.42	95 (± 1.50)	0.04	0.56
ds-DNA-A	0	0	0
ss-DNA-142	146,292 (± 48.0)	65.90	916.01
ss-DNA-14.2	14,902 (± 12.4)	6.71	93.27
ss-DNA-1.42	2,007 (± 0.42)	0.90	12.51
ss-DNA-A	0	0	0

* Aliquots (100 ul) of each solution were mixed with 10.0 ml Ready Safe scintillation cocktail and counted for 5 min.
** ds, double-stranded; ss, single-stranded; 142, 14.2, 1.42, nM of [^{14}C]HD used to treat DNA solutions; A, dry acetone.
@ Mean (± Standard Deviation) DPM of duplicate sample counts adjusted to 1.0 mg DNA. Based on ds-DNA A_{260}, 1 mg/ml = 20; ss-DNA A_{260} 1 mg/ml = 26.
# 1 nCi = 13.9 ng [^{14}C]HD.

Table II

SPECTROPHOTOMETRIC SCANS OF [^{14}C]HD-ALKYLATED DNA*

Preparation Code**	Wavelength, nm 280	260	230	260 nm / 280 nm+	230 nm / 260 nm+
ds-DNA-142	0.428	0.768	0.338	1.794	0.440
ds-DNA-14.2	0.433	0.788	0.348	1.820	0.442
ds-DNA-1.42	0.418	0.763	0.338	1.825	0.443
ds-DNA-A	0.438	0.793	0.353	1.811	0.445
ss-DNA-142	0.326	0.426	0.261	1.307	0.613
ss-DNA-14.2	0.321	0.456	0.296	1.421	0.649
ss-DNA-1.42	0.256	0.291	0.181	1.137	0.622
ss-DNA-A	0.156	0.226	0.116	1.449	0.513

* [^{14}C]HD-DNA samples adjusted to A_{269}, 1 mg/ml = 20 (ds-DNA) or 26 (ss-DNA) with Tris Buffer (see Materials and Methods section), and scanned automatically from 320 to 220 nm in a Shimadzu UV-260 spectrophotometer.
** ds, double-stranded; ss, single-stranded; 142, 14.2, 1.42, nM of [^{14}C]HD used to treat DNA solutions; A, dry acetone.
+ Corrected for background absorbance at 320 nm. Background averaged 0.022 ± 0.005 absorbance units for alkylated ds-DNA, and 0.114 ± 0.015 absorbance units for alkylated ss-DNA solutions.

Table III

SUMMARY OF ALKYLATION REACTIONS

A	B	C	D
[^{14}C]HD Treatment nM	x 10^{-3} mg [^{14}C]HD / Treatment *	x 10^{-3} mg [^{14}C]HD / Alkylated Preparation	Column C / Column B x 100 = % #
		ds-DNA-HD	
142.00	17.1370	1.0137	5.92
14.20	1.1230	0.0746	6.64
1.42	0.0092	0.0020	1.58
A	0	0	0
		ss-DNA-HD	
142.00	16.4020	1.4607	8.90
14.20	1.0751	0.1649	15.34
1.42	0.1227	0.0206	16.77
A	0	0	0

* mg of HD in treatment mixture (3.986 mg ds-DNA/4.7 ml; 3.816 mg ss-DNA/4.5 ml).
** Total HD in each alkylated DNA preparation (1 nCi = 13.9 ng [^{14}C]HD).
# Percent of treatment HD that alkylated DNA.

FIGURE 1.
[^{14}C]HD–Alkylated DNA Adducts

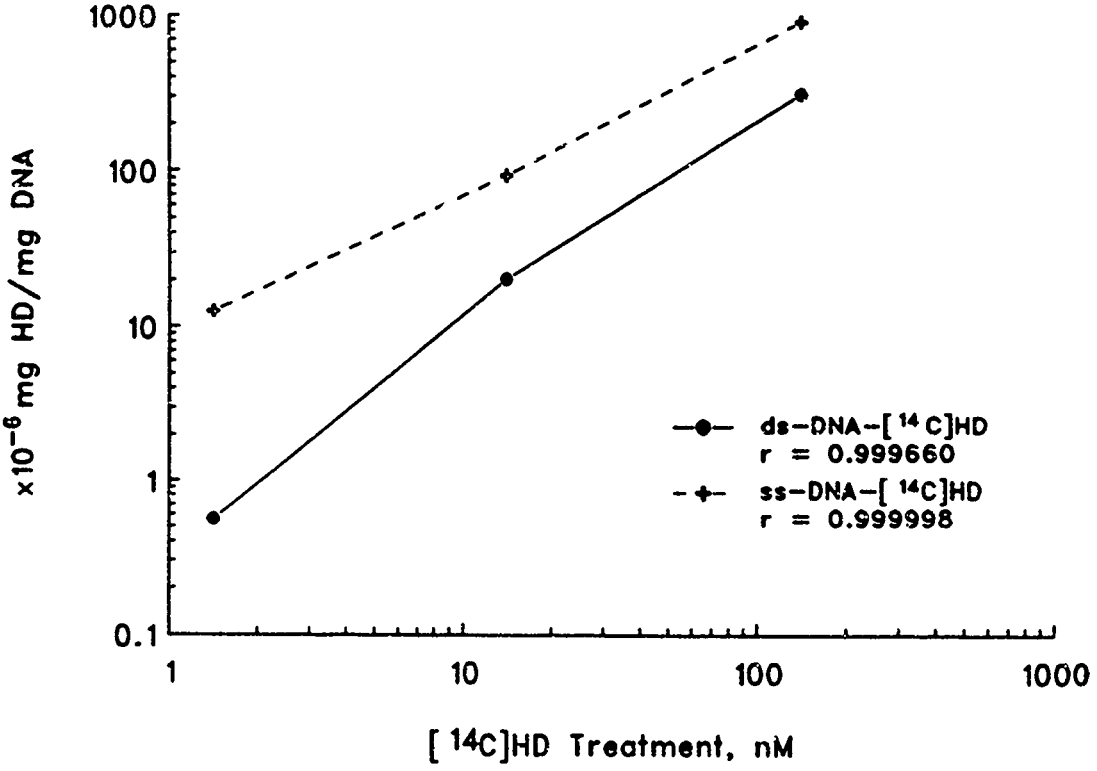
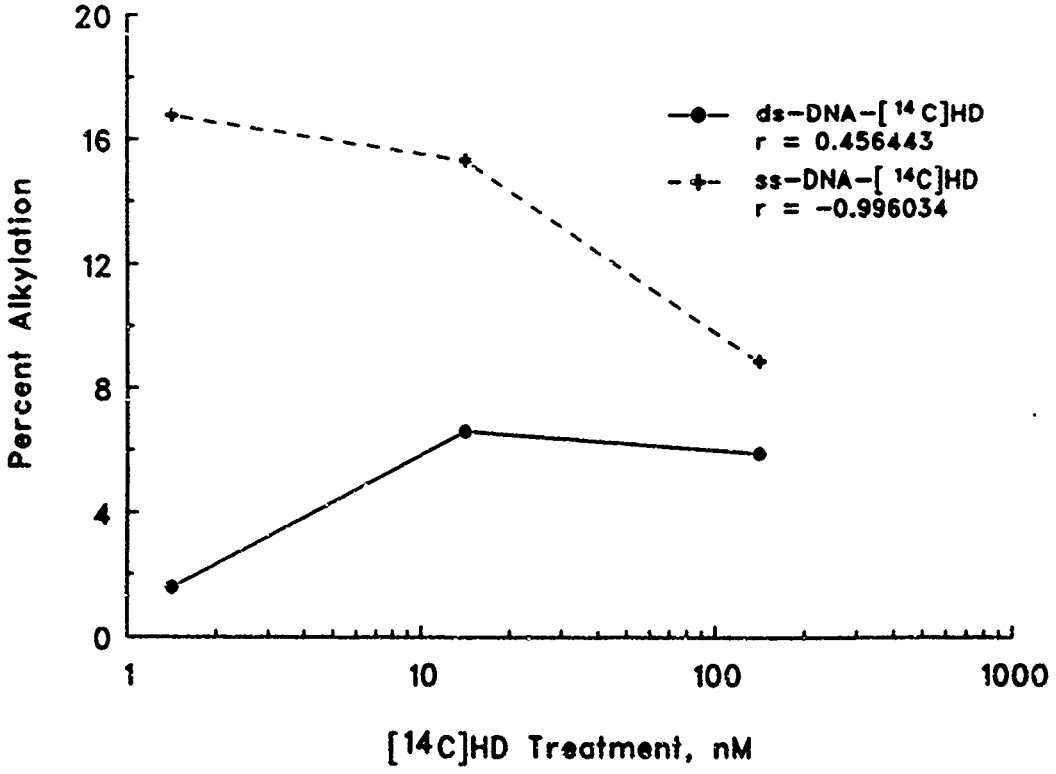


FIGURE 2.
[^{14}C]HD–DNA Alkylation Efficiencies



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APPENDIX

Treatment of Calf Thymus DNA with [^{14}C]HD by Mw. ir. A.G. Scheffer.

TREATMENT OF CALF THYMUS DNA WITH ^{14}C-HD

- Soak the double-stranded calf thymus DNA overnight in distilled water (>20 mg/ml)
- Dissolve the DNA (rotating overnight at room temperature) in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 7.4) to a concentration of about 2 mg/ml.
- Measure the amount of double-stranded DNA spectrophotometrically ($A_{260,1\ mg/ml}$= 20.
- Dilute the DNA-solution to a concentration of 1 mg/ml in PBS.
- Store the solutions in the refrigerator or freezer

Preparation of single-stranded DNA

- To induce single-strandedness, heat half the amount of the DNA-solution for 10 min at 100°C in closed heat-resistant vials.
- The solution was cooled down on ice.

DAY 1:

Treatment of calf thymus DNA with HD

- The double- and single-stranded DNA-solutions (1mg/ml) were divided in four equal portions for the treatment with HD (10, 100 and 1000 μM HD and untreated). Now you have 8 DNA-solutions.
- The HD was diluted in dry acetone in clean, dry, small vials as follows:
  100 mM HD-solution: 5 μl pure HD + 395.5 μl dry acetone
  This solution was diluted 1:10 and 1:100 (50 μl HD-solution + 450 μl dry acetone), resulting in solutions with a HD-concentration of 10 mM and 1 mM.
  These dilutions are 100 times more concentrated than the final concentrations for the treatment.
  Now you have 3 HD-solutions: 1, 10 and 100 mM.
- Add 0.01 volume of these three solutions to the DNA-solutions.
  (e.g. when you have 5 ml DNA-solution, add 50 μl HD-solution; so the final HD-concentrations are 10, 100 and 1000 μM and the final acetone concentration is 1%).
- Add to the untreated DNA-solutions only dry acetone to a final concentration of 1%.
- Stirr the DNA-solutions at the vortex.
- Incubate the 8 DNA-portions for 60 min at 37°C.

Purification of the DNA

- After 1 hr of incubation cool down on ice and keep the solutions on ice.
- Add 0.1 volume of cold 3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5 and shake.
- Add 3 volumes of -20°C absolute ethanol, now the DNA will precipitate.
- For the double-stranded DNA: collect the DNA with a glass pipet and wash the DNA in ice-cold 80% ethanol.
- Dry the DNA 2 min to the air.
- Dissolve the double-stranded DNA in 10 mM Tris-HCl, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$, 0.2 mM $ZnSO_4$, pH7.2 (about 1 mg/ml) overnight while rotating at room temperature.
- For single-stranded DNA: Incubate the DNA overnight at 4°C.

DAY 2:

- Measure the concentration of the double-stranded DNA spectrophotometrically ($A_{260,1mg/ml}$=20).
- Store the DNA-solutions at -20°C.
- Centrifuge the single-stranded DNA to the bottom of the vial (15 min; 4°C; 600g).
- Remove the supernatant and by a gentle stream of N_2 the last bit of alcohol is removed.
- Dissolve the DNA in 10 mM Tris-HCl, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$, 0.2 mM $ZnSO_4$, pH7.2 (about 1 mg/ml) overnight while rotating at room temperature

DAY 3:

- Measure the concentration of the single-stranded DNA spectrophometrically ($A_{260,1mg/ml}$=26 for single-stranded DNA).
- Store the DNA-solutions at -20°C.

MATERIALS:

- Deoxyribonucleic Acid from Calf thymus; Type I
  SIGMA, Chemical Company, St. Louis, USA
  No. D-1501, lot 67F-9730
  40 mg
- 100 ml PBS (0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 7.4)
- 50 ml dry acetone (HPLC grade)
- Molecular sive, type 3A or 4A
  for activation: heating overnight under vacuum at 250°C.
- 25 ml 3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5
- 200 ml -20°C absolute ethanol
- 200 ml 80% ethanol (H_2O:absolute ethanol = 2:8)
- 100 ml 10 mM Tris-HCl, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$, 0.2 mM $ZnSO_4$, pH 7.2

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